MARKED UP COPY OF PAGES 31, 32 AND 33 OF THE SEPTEMBER 11, 2003 AMENDMENT

claim and its dependant claim 215. Withdrawal of the rejections under 35 U.S.C. §112, second paragraph is respectfully requested.

IPE

Enablement Rejection – 35 U.S.C. §112, 1st paragraph

Claims 3, 171, 190 – 192, 196 – 199 remain rejected under 35 U.S.C. §112, first paragraph. Applicant respectfully traverse the rejections.

Applicant has amended the claims to further point out that the vector of the Applicant's invention is directed into a transcriptionally active intergenic spacer region of the chloroplast genome. Support for this amendment can be found on page 6, lines 11-15 and page 9, lines 31-35. Further, Sugita et al. (of record) demonstrated the presence of over 60 such spacer regions in plastid genomes.(Table II). This knowledge along with characterization of the plastid genome in a number of plants ~~[(Maier et al. 1995)~~], readily allows one skilled in the art to practice the Applicant's claimed invention. ~~*See*~~ Maier, Rainer et al., "Complete Sequence of the Maize Chloroplast Genomes: Gene Content, Hotspots of Divergence and Fine Tuning of Genetic Information by Transcript Editing", J. Mol. Biol, 251: (1995), *see* the discussion, pp. 614-626; see the discussion of the Maize Plastome, pg. 615, Col. 2, line 19 to pg. 618, Col. 1, line 13; *see also* Figure 1B, Length Comparison of Completely Sequenced Higher Plant Plastomes; Figure 2, Comparison Between Graminean Plants.

To further support the use of spacer regions for transgene expression, the Applicant has also included several examples of successful use of Applicant's invention. ~~For example, Hermann et al. (1999) (copy enclosed), the authors describe the targeting of transgenes into intergenic spacer region between *psbE* operon and a *petA* gene, which is known to be a suitable target site for the stable integration of transgenes. Hermann, Marita et al. "Transfer of Plastid~~

~~RNA Editing Activity To Novel Sites Suggests A Crtitical Role For Spacing In Editing Site Recognition" Proc. Natl. Acad. Sci. USA 96 (1999): 4856-4861; *see* pages 4858, lines 19-25.~~ [~~Another~~] An example is illustrated in ~~Ruf, Stephanie et al. "Stable Genetic Transformation Of Tomato Plastids And Expression Of A Foreign Protein In Fruit" Nature Biotechnology 19 (2001): 870 — 875~~. which used [~~yet~~] another spacer region of to transform tomato chloroplast. Ruf et al. 2001 illustrates the successful integration of transgenes between several tRNA Gly and tRNAfMet genes located between the psaB , psbC and psbD operon. *See id.* at page 874, column 2, lines 5 – 11. Furthermore, the Applicant has included, for the Examiner's convenience, a Table (citations omitted herein, but listed within the table) summarizing a multitude of spacer regions in which a number of foreign genes have been stably integrated into chloroplast genomes. As a result, the Applicant respectfully submits that these references support the use of any of a number of transcriptionally active spacer regions for the expression of transgenes and thus, one skilled in the art could predictably use any of a number of spacer regions to target transgenic expression.

Newly Amended Claims

Turning now to the Applicant's use of the phrase "either the 5' end or the 3' end, but not both" in the newly amended claims, Applicant respectfully submits that support for the use of such a phrase can be found in any of a number of examples described in the Applicant's specification, including Examples 2-16. Applicant submits that these transcriptionally active spacer regions are suitable for the insertion of transgenes without the need for 5' or 3' regulatory sequences, which can be expressed in the plastids of higher plants. One skilled in the art could readily use any identified spacer region to integrate foreign genes, without the use of regulatory sequence. Ruiz, Oscar et al. "Phytoremediation of Organomercurial Compounds Via Chloroplasts Genetic Engineering" Plant Physiol. 132 (2003): 1-9, *see* pg. 2, Col. 2, lines 24-29.

38-41. Applicant has shown several examples of stable integration and expression of foreign genes without using a 3' region or a promoter upstream of coding sequences, irrespective of the spacer region where foreign genes were integrated (rbcL/accD or trnI/trnA). In all of the expression cassettes described in the specification, the aadA gene has no 3' region and the gene of interest is without a promoter. These examples, which utilize regulatory elements of the plastid genome provide for the construction of a promoterless expression cassette wherein the gene coding for the peptide of interest can be driven by a native or an inserted promoter contained within the plastid genome but such a promoter is not present immediately upstream of the gene coding for the peptide of interest. The Applicant respectfully submits that a number of examples have illustrated that the gene coding for the peptide of interest does not contain a promoter directly upstream. Specifically, Figures 2A, 2B, 3A, 3B, 7B, 7D, 8, 25 and examples 1, 10, 11 and 16 show a gene of interest with no promoter directly upstream. The genes of interest, including genes conferring herbicide resistance, protein based polymer, insect resistance genes were expressed without a promoter. As another example, illustrating the use of such spacer regions with or without the need for 5' or 3' regulatory sequences, the Applicant has enclosed herewith a copy of Ruiz et al. (cited above) at page 169, Fig. 2B, wherein the pLDR-MerAB-3'-UTR and pLDR-MerAB vectors were constructed and successfully inserted into a spacer region of the plastid genome. [No difference was observed with or without the 3' UTR or with and without promoters]

Prior Art Rejection – Anticipation

Claims 3, 171, 190 – 192 remains rejected over various prior art references. Claim 192 has been cancelled. Rejections against claims 3, 171, 190 – 191 remain. Applicant respectfully traverses.

REMARKS

Transcription: Silent v. Active

In the Remarks (page 31, last paragraph, second sentence) starting with "For example..." and (to page 32, line 1) ending with "lines 19-25", on page 32, line 2, please consider this passage as deleted and the "Another" example should start with "An example..."The description of Hermann et al. (1999) that "the intergenic spacer region between *psbE* operon and the *petA* gene, which is known to be a suitable target site for the uptake trangenes." This is not an example of a transcriptionally active spacers. The invention deals with "transcriptionally active" regions. In the Description of the Invention, the invention distinguishes the "transcriptionally silent" region of the prior art Cannon et al. (Page 5, last paragraph). The description calls for the integration of a cassette into transcriptionally active region of the chloroplast chromosome". (See the description page 6, paragraph 1

The citation of Hermann et al. came about in that it was not realized that the authors were describing the intergenic silent spacer region between *psbE* operon and the *petA* gene. Page 31 is attached where the passage is crossed out.

Omission of the Terminal Sentence of the Passage on Page 33 of the Remarks About Ruiz et al.

The sentence "No difference was observed with or without the 3' UTR or with and without promoters" was unintentionally deleted before filing the Amendment in the PTO. That sentence has been reinserted.

Omitted Citation

On page 32 of the Amendment reference is made (on lines 8-9) to a "Table" for which a citation was omitted. The citation is "Chloroplast Genetic Engineering to Improve Agronomic Traits", authored by Henry Daniell, Oscar N. Ruiz and Amit Dhing, which was provided by the inventor, Henry Daniell by facsimile on September 19, 2003. (The manuscript is in press in "Methods in Molecular Biology"). The table referenced in the Amendment appears on pages 30, 31, and 32 and is discussed on pages 2 and 3 of the manuscript, respectively.

CONCLUSION

Counsel appreciates the recommendation of Examiner Fox made in a telephone conversation of September 23, 2003 with respect to the organization of this Amendment and to submit the entirety of the manuscript authored by Daniell et al. attached herewith.

The inventor and the undersigned thank Examiner Fox for the additional time spent on this case. We believe the case is in condition for allowance. If the Examiner has any questions, he is invited to call the undersigned at the telephone number shown below

Respectfully submitted,
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CLEAN COPY OF REMARKS SECTION FROM AMENDMENT
FILED SEPTEMBER 11, 2003

Applicant acknowledges and appreciates the Examiner's entrance of the Applicant's request for continued examination under 37 CFR 1.114 filed on December 16, 2002.

Interview Under 37 C.F.R. § 1.133, MPEP § 713.04

Applicant appreciates the personal interview granted by Examiner Fox on July 16, 2003 to Dr. Henry Daniell, the inventor, and his representative James Bauersmith in order to resolve the remaining issues in the case.

In light of the interview, the Applicant has amended the claims to resolve minor informalities and to further place the remainder of the claims in condition for allowance.

Non-Statutory Obvious-type Double Patenting Rejection

Applicant acknowledges with thanks the withdrawal of the Obvious-type Double Patenting Rejection over application Serial No. 08/972,901.

However, claims 3, 171, 190 – 192 are rejected under the judicially-created double-patenting doctrine over claims 19 – 23, 26 – 29, 31 and 34 of U.S. Patent No. 5,932,479. Applicant has addressed this rejection in a separate filing submitted on even date.

Indefiniteness – Rejections pursuant to 35 U.S.C. §112, 2d paragraph

Applicant acknowledges with appreciation the withdrawal of the indefiniteness rejections detailed in the previous office action. Likewise, acknowledges with appreciation the withdrawal of new matter rejections for claims 3, 171, 190, 191, 193 and 196 – 199.

Claims 193, 214 and 215 are rejected as being indefinite. By the foregoing amendments, Applicant has cured the indefiniteness of claim 190 by amending it to read "into a" in the penultimate line. Applicant has also deleted the reference to "which plant is the same as or different from the target higher plant" in claim 214, therefore curing the indefiniteness of that claim and its dependant claim 215. Withdrawal of the rejections under 35 U.S.C. §112, second paragraph is respectfully requested.

Enablement Rejection – 35 U.S.C. §112, 1st paragraph

Claims 3, 171, 190 – 192, 196 – 199 remain rejected under 35 U.S.C. §112, first paragraph. Applicant respectfully traverse the rejections.

Applicant has amended the claims to further point out that the vector of the Applicant's invention is directed into a transcriptionally active intergenic spacer region of the chloroplast genome. Support for this amendment can be found on page 6, lines 11-15 and page 9, lines 31-35. Further, Sugita et al. (of record) demonstrated the presence of over 60 such spacer regions in plastid genomes.(Table II). This knowledge along with characterization of the plastid genome in a number of plants readily allows one skilled in the art to practice the Applicant's claimed invention. Maier, Rainer et al., "Complete Sequence of the Maize Chloroplast Genomes: Gene Content, Hotspots of Divergence and Fine Tuning of Genetic Information by Transcript Editing", J. Mol. Biol, 251: (1995), *See the discussion* 614-626; see the discussion of the Maize Plastome, pg. 615, Col. 2, line 19 to pg. 618, Col. 1, line 13; *see also* Figure 1B, Length Comparison of Completely Sequenced Higher Plant Plastomes; Figure 2, Comparison Between Graminean Plants.

To further support the use of spacer regions for transgene expression, the Applicant has also included several examples of successful use of Applicant's invention. An example is illustrated in Ruf, Stephanie et al. "Stable Genetic Transformation Of Tomato Plastids And Expression Of A Foreign Protein In Fruit" Nature Biotechnology 19 (2001): 870 – 875 (copy enclosed) which used another spacer region of to transform tomato chloroplast. Ruf et al. 2001 illustrates the successful integration of transgenes between several tRNA Gly and tRNAfMet genes located between the psaB , psbC and psbD operon. *See id.* at page 874, column 2, lines 5

– 11. Furthermore, the Applicant has included, for the Examiner's convenience, a Table (citations omitted herein, but listed within the table) summarizing a multitude of spacer regions in which a number of foreign genes have been stably integrated into chloroplast genomes. As a result, the Applicant respectfully submits that these references support the use of any of a number of transcriptionally active spacer regions for the expression of transgenes and thus, one skilled in the art could predictably use any of a number of spacer regions to target transgenic expression.

Newly Amended Claims

Turning now to the Applicant's use of the phrase "either the 5' end or the 3' end, but not both" in the newly amended claims, Applicant respectfully submits that support for the use of such a phrase can be found in any of a number of examples described in the Applicant's specification, including Examples 2-16. Applicant submits that these transcriptionally active spacer regions are suitable for the insertion of transgenes without the need for 5' or 3' regulatory sequences, which can be expressed in the plastids of higher plants. One skilled in the art could readily use any identified spacer region to integrate foreign genes, without the use of regulatory sequence. Ruiz, Oscar et al. "Phytoremediation of Organomercurial Compounds Via Chloroplasts Genetic Engineering" Plant Physiol. 132 (2003): 1-9, *see* pg. 2, Col. 2, lines 24-29, 38-41. Applicant has shown several examples of stable integration and expression of foreign genes without using a 3' region or a promoter upstream of coding sequences, irrespective of the spacer region where foreign genes were integrated (rbcL/accD or trnI/trnA). In all of the expression cassettes described in the specification, the aadA gene has no 3' region and the gene of interest is without a promoter. These examples, which utilize regulatory elements of the plastid genome provide for the construction of a promoterless expression cassette wherein the gene coding for the peptide of interest can be driven by a native or an inserted promoter contained within the plastid genome but such a promoter is not present immediately upstream of the gene coding for the peptide of interest. The Applicant respectfully submits that a number of examples have illustrated that the gene coding for the peptide of interest does not contain a promoter directly upstream. Specifically, Figures 2A, 2B, 3A, 3B, 7B, 7D, 8, 25 and examples 1, 10, 11 and 16 show a gene of interest with no promoter directly upstream. The genes of interest, including genes conferring herbicide resistance, protein based polymer, insect resistance genes were expressed without a promoter. As another example, illustrating the use of such spacer regions with or without the need for 5' or 3' regulatory sequences, the Applicant has enclosed herewith a copy of Ruiz et al. (cited above) at page 169, Fig. 2B, wherein the pLDR-MerAB-3'-UTR and pLDR-MerAB vectors were constructed and successfully inserted into a spacer region of the plastid genome. No difference was observed with or without the 3' UTR or with and without promoters.

Prior Art Rejection – Anticipation

Claims 3, 171, 190 – 192 remains rejected over various prior art references. Claim 192 has been cancelled. Rejections against claims 3, 171, 190 – 191 remain. Applicant respectfully traverses.

As was discussed in the Examiner's interview, Applicant respectfully submit that Staub et al. (of record) does not teach the intergenic spacer region between the "universally" present *rbc*L and *acc*D chloroplast genes, because as is taught in Maier et al. (cited above), pg. 619, Figure 2B, the *rbc*L and *acc*D genes are not together in monocot chloroplast genomes. Specifically the *acc*D chloroplast gene does not exist in monocot chloroplast genomes, and is not universally present near *rbc*L in monocots.

The Authorities Relied Upon by the Examiner to Reject the Written Description of the Subject Invention Support the Applicant's Written Description of the Subject Invention

Applicant's attorney does not question the court's statement that *University of California v. Eli Lilly and Co.,* 119 F.3d 1559, 1568; 43 USPQ2d 1398, 1406 (Fed. Cir. 1997), "an invention requires precise definition, such as by structure, formula, [or] chemical name, of the claimed subject matter sufficient to distinguish it from other materials". In the instant application, the claims define the elements which comprise the universal integration and expression vector which comprises an expression cassette comprising a DNA sequence, both elements being defined for their function (coding, organization, etc.) The Examiner found but for some clarification in the claims, which are now corrected in this Amendment, that the claims defined the subject matter sufficiently to distinguish it from other material described in the prior art. Accordingly, the instant description is in accord with the *University of California* case.

The *MPEP* Section 2163, page 156 of Chapter 2100 of the August 2001 version, column 2, bottom paragraph, which the Examiner has relied upon, has been reviewed. Applicant's counsel submit that the DNA sequence is defined by its coding sequence for a peptide of interest, and the method of stable integration, whereby double homologous recombination, is facilitated. Therefore, the instant disclosure is in accordance with MPEP as cited above and the Guidelines published in *Federal Register*/ Vol. 66, No. 4/Friday, January 5, 2001/ Notices: pp. 1099-1111.

Likewise, *Amgen Inc. v. Chugai Pharmaceutical Co. Ltd.*, 18 USPQ 2d 1016 at 1021, (Fed. Cir. 1991) is relied upon for the statement that a gene is not reduced to practice until the inventor can define it by "its physical or chemical properties" (e.g. a DNA sequence). *Amgen,* which deals with a purification of Erythropoietin (EPO) is not pertinent to the claims of this application. The "cassette" is well defined by its elements, properties and function. The *University of California* case (citation omitted) has been discussed above.

The Examiner relies on MPEP 2111.02, *Pitney Bowes, Inc. v. Hewlett-Packard Co.*, 51 USPQ2d 1161, 1165 (Fed. Cir. 1999), *Rowe v. Dror*, 42 USPQ2d 1550, 1553 (Fed. Cir. 1997), and *In re Schreiber*, 44 USPQ2d 1429, 1431 (Fed. Cir. 1997) for the proposition that an intended use is not given patentable weight in product claims. Applicant respectfully asserts that the claim preamble, in its previous form, was necessary to give life, meaning and vitality to the claim in that it taught the universal applicability of the vector. Nevertheless, the current claims have now been amended to expedite prosecution. Claim 192 has been cancelled; therefore the rejection is moot.

Applicant acknowledges the availability of later publications as prior art under the limited circumstances that the publication serves to illustrate a universal fact. MPEP 2124 and 2131.01, part (III). However, as discussed above, Staub et al. (1995) does not "teach a region comprising the intergenic spacer region between the universally present *rbcL* and *accD* chloroplast genes" because the *acc*D chloroplast gene does not exist in monocot chloroplast genomes, and is not universally present near *rbc*L in monocots.

Chloroplast Genetic Engineering to improve agronomic traits

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Key Words: *Plastid transformation, GM crops, herbicide resistance, drought tolerance, disease resistance, phytoremediation, pest resistance/management, maternal inheritance, transgene containment.*

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1. Introduction

In the post-genomic era, nuclear and chloroplast genomes have been the targets of genetic manipulation in order to enhance the agronomic traits or introduce value-added traits into crop plants. Among several approaches, engineering the chloroplast genome is emerging as a successful approach. Manipulation of the chloroplast genome has become routine in the model system tobacco and has been extended to other edible solanaceous crops (potato and tomato). Expression of the transgenes in this organelle offers unique advantages that render this technology safe and acceptable to the public. Gene containment is the most notable advantage that this technology offers because chloroplast genomes are maternally inherited in most plant species (**1,2**). Besides chloroplast engineering overcomes the challenges of low level expression, gene silencing, position effect and multi step engineering of multiple genes that are current limitations of nuclear transformation (**3,4**). Chloroplast transformation has been used to express several biopharmaceutical proteins (**Table 1**) and engineer several agronomic traits (**Table 2**) that have been detailed in this review. In addition several reporter and selectable marker genes have been expressed via the chloroplast genome (**Table 3**).

1.1 Genome Organization and Principle of Chloroplast Transformation

Chloroplast genome is usually a circular molecule that is self-replicating and varies in size from 120 – 220 kb amongst different plant species (**5**). It is predominantly present as a single molecule but recent cytogenomic analysis has revealed that structural organization of chloroplast DNA is highly dynamic. It is arranged in both linear and circular conformation with 1-4 copies of the genome (**6**). A typical plant cell contains about 100 chloroplasts and each chloroplast further harbors approximately 100 copies of the same genome. This implies that a single gene is represented by at least 10,000 copies in a single plant cell. Single chloroplast genomes of most plant species possess two inverted repeat regions and thus copy number of the genes encoded by this region is about 20,000. Therefore, it is quite appealing to introduce a transgene into the chloroplast genome and obtain high levels of expression, taking advantage of the high copy number. Indeed several sites of insertion, including some in the inverted repeat region have been used for expression of foreign genes into the chloroplast genome **(see Tables 2,3)**. Site-specific integration of transgenes into the chloroplast genome differs from random nuclear integration because chloroplast transgenes are integrated via homologous recombination. Chloroplast transformation vectors are thus designed with homologous flanking sequences on either side of the transgene and introduced into the chloroplast via particle bombardment or using protoplasts by PEG treatment. Transformation is accomplished by integration of the transgene into a few genome copies initially followed by about 15-20 cell divisions under selection pressure thereby yielding homogenous population of plastid genomes. If the gene is introduced in the IR region, integration in one inverted repeat is followed by the phenomenon of copy correction that duplicates the introduced transgene into the other inverted repeat as well **(Figure 1)**. Chloroplast transformation vectors may also carry an origin of replication that facilitates replication of the plasmid inside the chloroplast thereby increasing the templates to be presented for homologous recombination and consequently enhancing the probability of transgene integration **(7, 8, 9)**.

2. Materials

2.1 Chloroplast transformation vector construction

1) Total cellular DNA from tobacco.
2) Primers designed to land on the chloroplast genome for the amplification of flanking sequences and the requisite regulatory elements like promoters and 5' and 3' untranslated regions.
3) Pfu-based DNA polymerase and dNTPs for PCR.

4) DNA modifying enzymes like T4 DNA polymerase for producing blunt-ends, Calf Intestinal Alkaline phosphatases to remove 5' and 3' phosphoryl groups from nucleic acids and T4 DNA ligase to form phosphodiester bonds.

5) PCR cloning kit.

2.2 Tissue culture and particle bombardment

1) Media: Murashige & Skoog salt mixture and Phytagar (Invitrogen), Sucrose, myoinositol, benzylaminopurine, napthaleneacetic acid (Sigma).

2) Particle bombardment: Particle gun – PDS 1000 He, Microcarriers – gold or tungsten particles, Macrocarriers, Macrocarrier holders, Rupture disks – 1100 psi (Biorad), Calcium chloride – biotech grade, Spermidine free base (Sigma). Working solutions of $CaCl_2$ and Spermidine free base as well as the handling of consumables for gene bombardment not listed in this work are prepared as explained in the chapter of this book titled, Stable Transformation of Plant Cells by Particle Bombardment/Biolistics.

2.3 Preparation of Tobacco Tissue Culture Media

MS medium

MS medium is used at several stages: for seed germination, plant propagation and root induction after the second round of regeneration on selection medium. MS medium is prepared by mixing a packet (4.3 g) of MS salts (Invitrogen corp., NY) and 30 g of sucrose in a 1L beaker and by adjusting the volume to 900 mL with distilled water. The pH is then adjusted to 5.8 with 1N KOH. Finally, the volume is made up to 1000 mL. The solution is placed in a 2 L flask and 6 g of phytagar is added at a final concentration of 0.6%. The solution is autoclaved for 20 minutes at 121 psi and allowed to cool to 40°C before adding any antibiotics to the medium. The growth medium is poured into deep Petri dishes, jars or magenta boxes.

RMOP medium

RMOP medium is used for shoot induction from tobacco leaves after they have been bombarded. This media contains phytohormones involved in the regeneration of shoots. The media is prepared by adding a packet of MS salts (4.3 g) (Invitrogen corp., NY), 30 g of sucrose, 100 mg of myo-inositol, 1 mL of a 100 mg/mL thiamine-HCl solution, 1 mL of a100 mg/ml BAP solution and 100 μL of a 100 mg/mL NAA solution in a 1 L beaker. The volume is adjusted to 900 mL and the pH is adjusted to 5.8 with 1N KOH. The volume is brought to 1000 mL with sterile distilled water or MilliQ grade water and phytagar is added to the medium at a final concentration of 0.6%. The medium is autoclaved for 20 minutes at 121 psi in a 2 liter flask (see **Note 1**). Finally, when the temperature of the medium drops below 40^0C, antibiotics may be added if the medium is to be used for selection. The medium is then poured into deep Petri dishes (100" x 25").

Spectinomycin solution

The spectinomycin solution is prepared by dissolving 1 g of spectinomycin into 10 mL of distilled water at a final concentration of 100 mg/mL. Then, the solution is filter sterilized under aseptic conditions in the fume hood and stored at –20°C.

2.4. Preparation of Gold Particles Suspension

1) The gold particles to be used for bombardment may be prepared in advance, at least one day prior to bombardment.
2) Weigh out 50 mg of gold particles (0.6 μm) into a 1.5 mL centrifuge tube.
3) Add 1 mL of 100% ethanol (molecular biology grade), vortex for 3 minutes.
4) Pellet the gold particles by centrifuging the tube for 2-5 minutes at maximum speed in a bench top microcentrifuge and then discard the supernatant.

5) Add 1 mL of 70% ethanol and vortex for 2 minutes.

6) Incubate the tube for 15 minutes at room temperature. Mix the contents of the tube about 3 times during the incubation.

7) Centrifuge the tube at maximum speed for 3 minutes, and then discard the supernatant.

8) Add 1 mL of distilled water and vortex for 1 minute or until particles are completely suspended.

9) Allow the particles to settle down for 1 minute at room temperature and then centrifuge the tube for 2 minutes; discard the supernatant.

10) Repeat steps 8 and 9 two additional times.

11) Add 50% (v/v) glycerol to the gold particles at a final concentration of 60 mg/mL.

12) Store the gold particles at –20°C until ready to use.

2.5. Working solutions for the plant bioassays

- 0.1 M cacodylate buffer pH 7.4: 2.5% glutaraldehyde, 2% paraformaldehyde and 5 mM $CaCl_2$
- 0.05 M glycine prepared in 1X PBS
- 2% glutaraldehyde diluted in 1X PBS
- 10 mM PMA working solution diluted in dimethyl sulfoxide
- glyphosate (0.5 to 5 mM)
- 3% or 6% PEG (MW 8,000, Sigma)

3. Methods

3.1 Amplification and cloning of flanking sequences

Flanking sequences are required for homologous recombination. It is desirable to amplify these with a polymerase that has proof reading properties. In our lab we use Pfu Turbo (Stratagene). Set up the polymerase chain reaction as follows, DNA: 100-200 ng; Buffer: 1X;

dNTPs: 200- 300 μM; Primer 1: 15-20 pmoles; Primer 2: 15-20 pmoles; Enzyme: 1- 2.5 units. Make up the total volume to 50 μl with sterile Milli Q (Millipore) grade water. The PCR is carried out by denaturing the PCR mix at 94^0C for 5 min followed by 30 cycles of denaturation at 94^0C for 30 sec, annealing at 55-60^0C for 30 sec and extension at 72^0C for 2-4 minutes as per the size of the expected PCR product. This is followed by an 8-10 minute extension at 72^0C. Treat the amplified DNA fragment with Taq polymerase in the presence of dATP in order to add A-overhangs that facilitate direct cloning of PCR products into pCR 2.1 cloning vector provided with the TOPO cloning kit (Invitrogen). For the addition of A-overhangs incubate the PCR product with dATP (200 μM), Taq DNA polymerase at 72^0C for 10 min. For cloning the DNA fragment with modified ends into pCR 2.1 vector follow the manufacturer's protocol (Invitrogen). The cloned fragment representing the flanking sequences is derived from the vector by digesting with appropriate restriction enzymes and is blunt-ended using T4 DNA polymerase following manufacturers protocol (NEB). Briefly, resuspend the DNA in 1X T4 DNA polymerase reaction buffer supplemented with 100 mM dNTPs. Add 1 unit T4 DNA polymerase per μg DNA and incubate for 15 min at 12^0C. Stop the reaction by adding EDTA to a final concentration of 10 mM and heating to 75^0C for 20 min. The blunt-ended fragment is then ligated with the help of T4 DNA ligase to PvuII digested pBluescript II KS dephosphorylated with Calf alkaline phosphatases as per manufacturer's instructions (Promega). For dephosphorylation, purify the digested DNA to be dephosphorylated by ethanol precipitation and resuspend the pellet in 40μl of 10mM Tris-Hcl (pH 8.0). Set up the following reaction: DNA (up to 10 pmol of 5'-ends): 40μl; CIAP 10X Reaction Buffer: 5μl; Diluted CIAP (0.01u/ml): up to 5μl. Make up the total volume to 50 μl with sterile Milli Q grade water (Millipore Inc.) Incubate the reaction at 37°C for 30 minutes and add another aliquot of diluted CIAP (equivalent to the amount used earlier) and continue incubation at 37°C for an additional 30 minutes. Finally add

300µl of CIAP stop buffer. Extract with phenol: chloroform and ethanol precipitate the DNA by adding 0.5 volume 7.5M ammonium acetate (pH 5.5) and 2 volumes of 100% ethanol to the final aqueous phase.

3.1.1. Constructing the chloroplast specific expression cassette

The basic chloroplast specific expression cassette is comprised of a promoter, selectable marker and 5'/3' regulatory sequences in order to enhance the efficiency of transcription and translation of the gene (**Figure 2**). The chloroplast specific promoters and regulatory elements are amplified from the total cellular DNA using specific primers designed on the basis of the sequence information available for the chloroplast genome of tobacco (Accession # NC_001879). The chloroplast specific expression cassette is cloned into a unique site in between the flanking sequences. Approximately 1 kb of homologous flanking regions is adequate to facilitate recombination. Care must be taken not to interrupt any genes while inserting the expression cassette. The site of insertion is therefore the intergenic spacer region.

3.2. Preparation for Bombardment

3.2.1. Preparation of Tobacco Tissues for Bombardment

The leaf material for particle bombardment is obtained from wild type tobacco plants, which are generated in two ways. Plants may be generated from seeds by germination of seeds in MS medium. The seeds are germinated in a Petri dish and then individual seedlings are moved to jars or magenta boxes containing MS medium. The time between seed germination and plant growth with the right size leaves is about two months. The leaves to be used in bombardment need to be green, with no damage or defects, and with an approximate size of 2" x 1". Another way to generate the plant tissue is by propagating nodal sections. In this method, nodal segments of aseptically grown plants are transferred to jars containing MS medium. This system decreases the time to obtain leaves of adequate size (see **Note 2**).

3.2.2. Preparation of consumables for bombardment

Consumables to be used during bombardment should be prepared in advance. Autoclave the macrocarrier holders, stopping screens, filter paper and Kimwipe. Sterilize the macrocarriers and rupture disks by submerging them in 100% ethanol for 10 minutes. Place the macrocarriers and rupture disks over autoclaved Kimwipes and dry them in the laminar flow hood.

3.3. Particle bombardment of Tobacco Leaves

3.3.1 DNA coating of the gold particles (see **Note 3**)

1) Vortex the previously prepared gold particles that were stored at –20°C until they are completely resuspended.
2) Pipette out 50 μl of the gold particle suspension into a 1.5 ml microcentrifuge tube.
3) Add 10 μL of plasmid DNA that has a concentration of 1 μg/μL, vortex for 5 seconds.
4) Add 50 μL of freshly prepared filter sterilized 2.5 $CaCl_2$, and vortex for 5 seconds.
5) Add 20 μl of 0.1 M spermidine-free base and vortex for 5 seconds.
6) Vortex the mixture for 20 minutes at 4°C.
7) Add 200 μL of room temperature absolute ethanol to the mixture, vortex for 5 seconds and then centrifuge the mixture for 30 seconds at 3000 rpm. Remove the supernatant and repeat step 7 four times.
8) After the final step, resuspend the pellet in 30 μl of 100% ethanol.
9) Keep the particles on ice until ready to use.

3.3.2. Tobacco leaf samples for bombardment (see **Note 4**)

1) Take five fully expanded and undamaged green leaves from a young wild type tobacco plant growing in a jar or a magenta box.

2) Place an autoclaved Whatman filter disc on RMOP medium and a leaf on it with its adaxial side facing the medium. The abaxial side is the one that is to be bombarded (see **Note 5**).

3) Cover the petridish and repeat for all the leaves.

3.3.3. Macrocarrier loading (see Note 4)

1) The macrocarrier is placed inside the macrocarrier holder with its concave side facing outward. Use the macrocarrier insertion tool in order to push the macrocarrier in place inside the holder.

2) Resuspend the gold particles completely by vortexing and pipetting in order to eliminate any clumps. Aliquot 5 μL of the gold particles coated with the plasmid DNA (microcarrier) and spread it out in the center of the macrocarrier.

3) Repeat steps 1 and 2 with additional macrocarriers based on the number of leaves to be bombarded.

4) DNA coated gold particles remaining after loading all the macrocarriers may be used for a second application on the macrocarriers, already containing the gold particles. Alternatively, remaining gold particles coated with DNA could be run on agarose gels to test DNA binding efficiency.

3.3.4. Particle Bombardment (see Note 4, see Note 6)

The particle bombardment procedure explained in the chapter of this book entitled, Stable Transformation of Plant Cells by Particle Bombardment/Biolistics may be followed. Exceptions to this procedure are pointed below.

1) Using the adjustment handle of the helium regulator, rotate it clockwise until the pressure is set at 1350 psi.

2) Place a rupture disk (1100 psi) into the rupture disk-retaining cap and rotate it into position in the gene gun tightly.

3.4 Tobacco Tissue Regeneration and Selection (see **Note 7**)

1) Incubate bombarded leaves in the dark for 48 hours at 27°C.
2) After 48 hours, cut leaves into small pieces of about 5 mm^2 each with the help of sterile scalpel blade.
3) Place the small pieces on agar containing RMOP medium (in Petri dish 100" x 25") supplemented with 500 μg/mL spectinomycin antibiotic for selection; this is called the first round of selection. Place the bombarded (abaxial) side of the leaf in direct contact with the selection medium. RMOP medium induces shoot formation.
4) Incubate the leaf tissues at 27°C in a photoperiod of 16 hours light and 8 hours dark.
5) First putative transgenic shoots may be obtained within 4 to 6 weeks after placing the bombarded leaf sections on the selection medium.
6) Once the putative transgenic shoot obtained in the first round of selection starts developing leaves that are about 1 cm^2 in size, small sections of these are placed on new RMOP medium supplemented with spectinomycin for second round of selection.
7) For the second round of selection, cut the leaf into pieces of about 2 mm^2 size and place them on RMOP medium containing suitable antibiotic as the selection agent. Again, ensure that the abaxial side of the leaf is in direct contact with the medium.
8) These leaf sections will produce transgenic shoots in about 3-4 weeks. Once the shoot develops into a small plantlet, it is detached from the leaf tissue and transferred to MS agar medium supplemented with spectinomycin. This step is termed as the third round of selection where root formation occurs.

9) After about 4-6 weeks in the rooting medium, the plant is completely developed and ready to be moved to soil in pots.

10) To transfer the plant to soil, gently pull out the plant from the agar and wash roots thoroughly with tap water in order to remove any agar attached to the roots.

11) Grow plants in high nutrient soil at appropriate temperature 16h/8h light/dark photoperiod in a growth chamber or a green house.

12) Collect seeds from T0 generation transgenic plants.

3.5 Molecular and Biochemical Analyses of transformed plants

3.5.1. PCR analysis

PCR analysis is used to screen the transgenic plants and distinguish true chloroplast transgenic plants from mutants or nuclear transgenic plants. Site-specific chloroplast integration of the transgene cassette is determined by using a set of primers one of which anneals to the native chloroplast genome and the other anneals within in the transgene cassette. Mutant and nuclear transgenic plants are not expected to produce a PCR product with these primers. Chloroplast specific integration of the transgene cassette can be further confirmed by using a set of primers that anneal to flanking sequences used for homologous recombination. The size of the PCR product should then depend on size of the transgene cassette. This initial screening is very important for eliminating the mutants and nuclear transgenic plants from the transgenic plant population.

1) Isolate total DNA from wild type and transgenic plants using DNeasy™ Plant Mini Kit (QIAGEN, inc, Valencia, CA) and use this DNA as a template for PCR reactions.

2) In a 50 μL PCR reaction add: 1 μL of 100 ng/μl genomic DNA, 5 μL of 10X PCR reaction buffer, 1 μL each of 10 μM 5' forward and reverse primers, 200-250 μM each of dNTPs, 0.5 μL (2.5 units) Taq DNA polymerase and sterile distilled water.

3) Perform the polymerase chain reaction under following reaction conditions: Denaturation for 5 minutes at 94°C, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 60-65°C for 1 minute, extension at 72°C for 1 minute. The time of extension can be varied depending on the size of the amplicon. Usually 1 minute per kb is the prescribed elongation time for most DNA polymerases. This is followed by a final step of 7-10 minutes of elongation at 72°C.

4) Detect PCR amplification products in agarose gels by electrophoresis and stained with ethidium bromide (EtBr).

3.5.2. Southern analysis

Southern analysis is performed to determine the copy number and degree of homoplasmy of the introduced transgene. A single plant cell harbors about 10,000 copies of the chloroplast genome. Initially, the transgene cassette integrates into a few of these genomes and under selection pressure its copy number gradually increases. After three rounds of selection, the untransformed genome copies are replaced by the transgenic genome copies, leading to complete homoplasmy. This can be ascertained by Southern analysis of the total DNA isolated from plants generated after the third round of selection. The southern blot is probed with radiolabeled flanking sequences used for homologous recombination. The transgenic plants possess higher molecular weight plastid genome that is distinguishable on the autoradiogram from the smaller molecular weight fragment representing the untransformed plastid genome. If the transgenic plants are heteroplasmic, a wild type fragment is visible along with the larger transgenic fragment. Absence of the wild type fragment confirms the establishment of homoplasmy (see **Note 8**).

3.5.3. Bioassays

Bioassays assess the effectiveness or properties or functionality of the introduced transgene (s). Additionally, transgenic plants may be exposed to more rigorous challenges than what they would normally encounter in the real environment. Bioassays provide an accurate estimate on the new capabilities conferred to the plant by the integrated transgene (s).

Insect resistance

Cry2Aa2 is one of the insecticidal proteins produced by the bacterium *Bacillus thuringensis*. Genetically modified plants with insecticial proteins have shown significantly increased resistance against insects. Cry2Aa2 protein is encoded by an operon and has been expressed via chloroplast genome as a single gene **(10)** or as an operon **(11)** that contains the cry2Aa2 gene and the ORF 2, which is a chaperone that folds the Cry2Aa2 protein into crystals. DeCosa et al (**11**) demonstrated the formation of cuboidal crystals when the complete cry2Aa2 operon was expressed. In addition to the ORF 2 protein, crystal formation was also facilitated by hyper expression of the insecticidal protein through chloroplast genetic engineering. Chloroplast transgenic plants showed that Cry2Aa2 accumulated up to 46.1% of total protein and this is the highest reported level of foreign protein expression in transgenic plants. Transgenic plants expressing the single cry2Aa2 gene or the complete cry2Aa2 operon showed high insecticidal activity when compared with wild type tobacco plants. When non-transgenic control tobacco leaves were fed to the tobacco budworm (*Heliothis virescens*), cotton bollworm (*Helicoverpa zea*) and beet armyworm (*Spodoptera exigua*), the leaf pieces were completely consumed after 24 hours. When the tobacco budworm was fed with a leaf from transgenic plants expressing the single gene, it died after 5 days while the insect fed with the leaf from the plant expressing the operon died in 3 days (**Figure 3**). Similar results were obtained when the assays were repeated with the cotton bollworm and the beet armyworm. This proved that the hyper expression of the cry2Aa2 gene through chloroplast engineering of the cry2Aa2 gene can confer 100% resistance to insects that feed on transgenic plants. Most importantly, chloroplast transgenic plants killed insects that were 40,000-fold resistant to insecticidal proteins. It was also observed that the old senescent leaves contained very high levels of the insecticidal protein, inspite of high protease activity. This could be attributed to chaperone assisted crystal formation that prevented proteolytic degradation of the protein and allowed its higher accumulation. Indeed electron microscopic analysis revealed the presence of cuboidal crystals of the insecticidal protein in mature and old chloroplast transgenic leaves expressing the cry2Aa2 operon (**Figure 4**).

Insect bioassay

1) Cut 2 cm^2 leaf disks from transgenic and wild type plants.
2) Place the leaf segments over distilled water-soaked cardboard lids in 50 × 12 mm plastic Petri dishes with tight-fitting lids.
3) Add 5 to 10 neonate insects (*Heliothis virescens, Helicoverpa zea, Spodoptera exigua*) per sample, with three replicates per treatment.
4) Examine the insect mortality daily for a period of 5 days.
5) Repeat the experiment at least thrice, but preferably 4 to 5 times.
6) As a control, include a leaf disk from another chloroplast transgenic plant harboring only the basic transformation cassette devoid of the cry2Aa2 gene. This would confirm that the observed results are actually due to the insecticidal protein and not due to some other extraneous factors.

Transmission electron microscopy and immunogold labeling

1) Cut the transformed and untransformed leaf into 1-3 mm^2 sections.
2) Fix sections in 0.1 M cacodylate buffer pH 7.4 for 15 minutes under vacuum and 12 hours at 4°C.
3) Rinse the samples twice in 0.1 M cacodylate (pH 7.4) after fixation.

4) Dehydrate fixed samples through a graded ethanol series to 95%, then implant in LRW resins at 60°C for 24 hours.

5) Cut ultra-thin sections using a Leica Ultracut T ultramicrotome and collect section onto nickel grids

6) Incubate sections in 0.05 M glycine prepared in PBS for 15 minutes to inactivate residual aldehyde groups.

7) Place grids onto drops of blocking solution (PBS containing 2% non-fat dry milk) and incubate for 30minutes.

8) Incubate sections for 1 hour in rabbit anti-Cry2A polyclonal antibody (dilution range from 1:1000 to 1:25,000 in blocking solution).

9) Wash sections with blocking solution 6 times for 5 minutes each.

10) Incubate sections for 2 hours with a goat anti-rabbit IgG secondary antibody conjugate to 10 nm gold diluted 1:40 in blocking solution.

11) Wash sections 6 times for 5 minutes each in blocking solution and 3 times for 5 minutes each with PBS. Fix sections in 2% glutaraldehyde for 5 minutes.

12) Wash fixed section in PBS 3 times for 5 minutes each, then in distilled water 5 times for 2 minutes each.

13) Stain sections using uranyl acetate and lead citrate and observe samples under transmission electron microscope at 60 kv.

Phytoremediation

Mercury and its most toxic form, organomercurials, present a serious hazard to the environment and ecosystems. Chemical and physical remediation procedures as well as bacterial bioremediation methods have proven ineffective due to the high cost and environmental concerns. As an alternative, phytoremediation has been proposed as a system for safe and cost efficient remediation of toxic chemicals in the environment. Mercury and organomercurials mainly target the chloroplasts. For this reason it is advantageous to use chloroplast genetic engineering to increase resistance to mercury and organomercurials and at the same time detoxify the highly toxic organomercurials and metal mercury forms present in the contaminated environment (**12**). To achieve this, two bacterial enzymes that confer resistance to different forms of mercury known as mercuric ion reductase and organomercurial lyase were over expressed in the chloroplast through chloroplast genetic engineering. When the chloroplast transgenic plants containing the operon with the mercuric ion reductase and organomercurial lyase were tested through a bioassay in which the extremely toxic organomercurial, phenyl mercuric acetate (PMA) was used, the transgenic plants were substantially more resistant than wild type tobacco plants growing under the same conditions. The 16 days old tobacco plants (seedlings) were able to grow well in soil containing PMA concentrations of 50 μM, 100 μM and even survived at the highest concentration of 200 μM. On the other hand, wild type tobacco plants struggled to survive at concentrations of 50 μM PMA (**Figure 5**). When nuclear transgenic tobacco seeds containing the *mer*A and *mer*B genes were germinated in medium containing PMA, they were only resistant to concentrations of 5 μM PMA (**13**). 24 day-old chloroplast transgenic plants treated with concentrations of 100, 200, 300 and 400 μM PMA showed an increase in the total dry weight when compared with wild type growing at the same concentrations. On the other hand, the total dry weight of wild type plants progressively decreased with each increase in PMA concentration from 0 to 400 μM. Chlorophyll content of the leaf is an indication of the chloroplast structural and physiological integrity. When 15 mm diameter leaf discs from wild type and transgenic plants were grown for 10 days in a concentration of 10 μM PMA, the chlorophyll concentration of the transgenic plants increased, while the wild type was reduced. These bioassays show the efficiency and activity of the chloroplast expressed enzymes and establishes that chloroplast genetic engineering can be used for phytoremediation. This is the first report of the use of chloroplast genetic engineering for phytoremediation

Organomercurial bioassay

1) Surface-sterilize tobacco seeds in 7% sodium hypochlorite containing 0.1% Tween 20. Vortex the tube for 5 minutes, then wash the seeds five times with distilled water. Finally dry the seeds in a speed vac at medium temperature.
2) Germinate the transgenic sterilized seeds in a plate containing one-half strength Murashige and Skoog medium with suitable selection agent and 0.3% phytagar. Adjust pH to 5.7 with 1N KOH. The wild type seeds are germinated in the same media but lacking selection agent.
3) Incubate plates with seeds at 4°C for 3 day, and then transfer to a growth chamber in a 16 hours light photoperiod at a temperature of 24°C, humidity of 75% to 90% and a photon flux density of 750 $\mu E/m^2$.
4) Transfer seedling to soil (sand:Davis Mix, 50:50) approximately 10 days after germination and maintain in the greenhouse at 22°C using a 16 hour light photoperiod.
5) Five replicate pots each containing a single seedling (wild type and transgenic) is used for the assay.
6) Water the pots twice a week with one-half-strength Hoagland solution.
7) Six days after the initial transfer of seedlings into soil, apply three different concentrations of PMA to the pots containing wild type and transgenic plants, in three replicates for each of the concentrations.
8) Prepare a 10 mM PMA working solution.

9) Add 100 mL of one-half-strength Hoagland solution containing concentrations of 50, 100 and 200 μM PMA. Control receives the same treatment but without PMA.

10) Grow plants under conditions as explained in step 4 for at least 14 days. Then take picture and assess total biomass by measuring the length of the root and shoot as well as root and shoot total dry weight.

Disease resistance

Helical structured antimicrobial peptides (AMPs) are expressed as protective agents against pathogens in many organisms. We have expressed MSI-99, an analog of magainin 2 in transgenic chloroplasts **(14)**. This AMP confers protection against prokaryotic organisms due to the high specificity for negatively charged phospholipids, which are mostly found in bacteria and less abundant in eukaryotic organisms. *In planta* bioassay was performed with chloroplast transgenic plants expressing MSI-99. The leaves were inoculated with the phytopathogen *P. syringae pv tabaci* and the absence of necrosis around the inoculation area demonstrated increased resistance to pathogen colonization and infection. No necrotic tissue was observed in transgenic plants even when 8 x 10^5 cells were inoculated. When wild type plants were inoculated with 8 x 10^3 cells of the same phytopathogen (a much lower number of cells than in the cells used for transgenic plants), a large necrotic area was observed **(Figure 6 C, D)**. This suggests that high levels of AMP are expressed by the chloroplast and that this is released from the chloroplast during pathogen infection. Studies of bacterial population at the site of inoculation 4 days after inoculation showed that wild type plants had a cell population of 13,750 ± 750CFU compared to the lower count in transgenic plants of 4,650 ± 125 CFU. When similar bioassays were performed with the plant pathogen, yeast *Colletotrichum destructivum* in non-transformed controls, the plant developed anthracnose lesions whereas transgenic plants expressing MSI-99 did not develop any lesions **(Figure 6 A, B)**. This study shows that chloroplast genetic engineering can be used to confer high level resistance to phytopathogenic organisms in plants.

In planta bioassay

1) Grow a culture of *P.syringae pv tabaci* overnight (ATCC 17914) in liquid nutrient broth. Alternatively, grow *Colletotrichum destructivum* (ATCC 42492) in Czapek yeast autolysate agar at 24°C.
2) Centrifuge the *P.syringae pv tabaci* culture and resuspend the pellet in 50 ml of 0.01 M phosphate buffer. Make dilutions of the suspension in phosphate buffer. Obtain *Colletotrichum destructivum* by flooding the agar plate with 9 mL of distilled water and remove the spores aseptically. Dilute the inoculum to a final density of approximately 1×10^6 spores mL^{-1}.
3) Prepare the leaf by scraping the leaf with fine-grain sandpaper to an area of 7mm.
4) Add 10 μl of 8×10^5, 8×10^4, 8×10^3 and 8×10^2 cell culture of *P. syringae pv tabaci* to each prepared area in transgenic and wild type plants. To inoculate *Colletotrichum destructivum*, place 8 drops containing 10 μL of the diluted inoculum.
5) Take photograph 5 days after inoculation.
6) In another assay use 25 ml of 8×10^5, 8×10^4, 8×10^3 and 8×10^2 cell culture of *P. syringae pv tabaci* and inject it into the leaf of wild type and transgenic tobacco plants by using a needle with a syringe.
7) Take photograph 5 days after inoculation.
8) Collect leaf disks containing the inoculated area from transgenic and wild type plants, 4 days after inoculation.
9) Grind the samples in 300 μl of 10 mM $MgCl_2$.
10) Transfer the homogenates to 5 mL PO_4 buffer.

11) Plate dilutions of the samples in *Pseudomonas* Agar F (Difco) for 48 hours at 28°C.

12) Enumerate colonies.

Herbicide resistance

Glyphosate is a broad-spectrum herbicide that kills majority of grasses and broad leaf weeds. Glyphosate acts by competitive inhibition of the 5-enol-pyruvyl shikimate-3-phosphate enzyme (EPSPS). This disrupts the aromatic amino acid biosynthetic pathway that only occurs in plants and microorganisms. Because this potent herbicide lacks selectivity, for successful weed control in crop plantations, the crops have to be genetically modified to resist glyphosate. Usually the targets for most herbicides, including glyphosate, are amino acids and fatty acid bioasynthetic pathways found in the chloroplast. We have shown that the hyper expression of the petunia EPSPS (which is highly sensitive to glyphosate) through chloroplast transformation conferred resistance to high levels of glyphosate and the transgene was maternally inherited (**8**). When concentrations of up to 5 mM Glyphosate were sprayed on chloroplast transgenic plants expressing the petunia EPSPS, they survived without any detrimental symptoms (**Figure 7**). Untransformed tobacco plants were highly susceptible to glyphosate, dying 7 days after exposure to 0.5 mM glyphosate. More recently, tobacco plants were transformed via chloroplast genetic engineering with more resistant forms of EPSPS, including AroE (Bacillus) and CP4 (Agrobacterium, **15**).

Glyphosate tolerance test

1) Spray wild type and transgenic plants growing in soil with equal volumes of different concentrations of glyphosate (0.5 to 5 mM).
2) Take picture each week after initial exposure to glyphosate.

Maternal inheritance

Transgenes integrated into chloroplast genomes are, in general, inherited maternally. This is evident when transgenic seeds (as shown in **Figure 8**) are germinated on RMOP basal medium containing 500 μg/mL spectinomycin. There should be no detrimental effect of the selection agent in transgenic seedlings whereas untransformed seedlings will be affected. In Figure 8 B, all transgenic seedlings carry the spectinomycin resistance trait and show maternal inheritance without any Mendelian segregation of introduced transgenes. If further confirmation is necessary, pollen from chloroplast transgenic lines may be used to fertilize wild-type untransformed plants; progeny should not carry the trait, if they were maternally inherited.

Drought tolerance

Environmental stress factors like drought, salinity or freezing are mostly hazardous to plants due to their sessile way of life. Osmoprotectants are produced in plants, yeast, and other organisms, and this confers resistance to several factors including drought. The TPS1 gene from yeast encodes the trehalose phosphate synthase, an enzyme that produces the osmoprotectant trehalose. Attempts to confer resistance to drought by expressing this enzyme via nuclear transformation have proven ineffective due to adverse pleotropic effects even at very low levels of trehalose accumulation. We reported the hyper expression of the trehalose phosphate synthase and the increased accumulation of trehalose in chloroplasts of transgenic plants (**16**). When TPS1 was expressed through chloroplast transformation, no pleotropic effects were detected and the plant was as healthy as wild type controls (**Figure 9**). Drought tolerance bioassays in which transgenic and wild type seeds were germinated in MS medium containing concentrations of 3% to 6% polyethylene glycol (PEG) showed that the chloroplast transgenic plants producing high levels of trehalose, germinated, grew, maintained green color and remained healthy (**Figure 10**). Wild type seeds germinated under similar conditions showed severe dehydration, loss of chlorophyll (chlorosis), and retarded growth that finally ended in the death of the seedlings. Loss of chlorophyll in the non-transgenic plants reveals that drought affects thylakoid membrane stability. Production of trehalose in the chloroplast of transgenic plants conferred membrane stability. In another assay, when seedlings from transgenic and wild type tobacco plants were dried for 7 hours, they showed dehydration symptoms, but when the seedlings were re-hydrated in MS medium for 48 hours, all chloroplast transgenic plants accumulating trehalose recovered and grew fine. The wild type controls became bleached and died (**Figure 10**). Additionally, when potted transgenic and wild type plants were not watered for 24 days and were then rehydrated for 24 hours, the transgenic plant recovered while the control plant did not recover. These results show that expression of the enzyme trehalose phosphate synthase via chloroplast genetic engineering confers resistance to drought.

Drought tolerance bioassays

PEG bioassay

1) Germinate sterilized chloroplast transgenic and wild type tobacco seeds in MS medium plates containing 3% or 6% PEG (MW 8,000, Sigma).
2) Take a photograph 4 weeks after plating the seeds.

Dehydration / rehydration assay

1) Germinate chloroplast transgenic and wild type tobacco seeds on agarose with or without 500 μg/ml spectinomycin, respectively.
2) Take three-week-old seedlings from transgenic and wild type and air-dry for 7 hours at room temperature in 50% relative humidity.
3) Rehydrate for 48 hours by introducing the seedlings root into MS medium.
4) Place rehydrated seedlings in MS media plate and allow them to grow for several days.
5) Compare wild type and transgenic plants. Take picture.

4. Notes

1. It is important not to autoclave RMOP medium for more than 30 minutes because the phytohormones added to the medium may break down, inhibiting tissue regeneration.
2. It is of importance to note the number of times a plant is propagated. We have noted that if a plant is propagated more than 5 times, the transformation efficiency of the leaf decreases. Additionally, if the plant to be used for bombardment is flowering, the plant is senescent. This is a common problem with certain varieties like Petit Havana. We have also noted that this also decreases the transformation efficiency of the plant.
3. The order of adding gold, DNA, $CaCl_2$, and spermidine is essential for the proper coating of the gold particles. $CaCl_2$ should be prepared fresh and the DNA coated gold particles have to be used within 2 hours.
4. The preparation of leaf tissues, macrocarriers and the bombardment has to take place under aseptic conditions in a laminar flow hood. Before each addition of gold particles into the macrocarrier, make sure to vortex the particles for at least 30 seconds to resuspend, and use immediately. It is essential to avoid clumps of gold particle when loading the macrocarriers because this will damage the leaf tissue during bombardment and decrease transformation efficiency.
5. The side of the leaf to be bombarded has to be the abaxial side because it does not contain the waxy cuticle found in the upper side of the leaf. This allows for better penetration of the gold particles and increases transformation efficiency. It is essential to cut the leaf as close to the time of bombardment as possible, this helps in decreasing the activation of nucleases and proteases (often detected in detached leaves) that could affect the transformation process.
6. The microcarrier launch assembly has to be placed in level one (L1=3 cm), meanwhile the target plate shelf has to be placed in level four (L4=12 cm). The stopping screen

support has to be placed in between the spacer rings (one under and one over the stopping screen). This setup is essential for efficient chloroplast transformation.

7. When placing the leaf pieces on RMOP agar medium containing the selection agent, make sure to leave enough space in between pieces (we recommend about 5 pieces per plate). This will allow the full expansion of the leaf segments. First round of selection is an appropriate time to screen for integration of transgenes into the chloroplast genome by PCR. When moving the transgenic plants to soil from third round of selection, it is essential to remove any agar attached to the roots. This will decrease the possibility of fungal and bacterial contamination when grown in soil.
8. The presence of the foreign gene into the nuclear or mitochondrial genome can be detected by using the foreign gene as a DNA probe and prolonged exposure of blots on films.

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Table 1

Agronomic traits	**Gene**	**Site of Integration**	**Promoter**	**5'/3' Regulatory elements**	**Ref**
Insect resistance	Cry1A(c)	*trn*V/*rps*12/7	Prrn	*rbc*L / T*rps*16	**17**
Herbicide resistance	CP4 (petunia)	*rbc*L/*acc*D	Prrn	ggagg / T*psb*A	**8**
Insect resistance	Cry2Aa2	*rbc*L/*acc*D	Prrn	ggagg (native) / T*psb*A	**10**
Herbicide resistance	CP4 (bacterial or synthetic)	*trn*V/*rps*12/7	Prrn	*rbc*L or T7 gene 10 / T*rps*16	**15**
Insect resistance	Cry2Aa2 operon	*trn*I/*trn*A	Prrn	Native 5'UTRs / T*psb*A	**11**
Disease resistance	MSI-99	*trn*I/*trn*A	Prrn	ggagg / T*psb*A	**14**
Salt and drought tolerance	*tps*	*trn*I/*trn*A	Prrn	ggagg / T*psb*A	**16**
Phytoremediation	*mer*A[a]/*mer*B[b]	*trn*I/*trn*A	Prrn	ggagg[a, b] / T*psb*A	**12**

Table 2

Biopharmaceutical proteins	**Gene**	**Site of Integration**	**Promoter**	**5'/ 3' regulatory elements**	**% tsp expression**	**Ref**
Elastin derived polymer	EG121	*trn*I/*trn*A	Prrn	T7gene10 / T*psb*A	ND	**9**
Human somatotropin	*hST*	*trn*V/*rps*12/7	Prrn^{a}, P*psb*A^{b}	T7gene10^{a} or *psb*A^{b} / T*rps*16	7.0 %^{a} and 1.0%^{b}	**18**
Cholera toxin	*Ctx*B	*trn*I/*trn*A	Prrn	Ggagg / T*psb*A	4%	**19**
Antimicrobial peptide	MSI-99	*trn*I/*trn*A	Prrn	Ggagg / T*psb*A	Not tested	**14**
Insulin like growth factor	*IGF*-1	*trn*I/*trn*A	Prrn	P*psb*A/T*psb*A	33%	**20**
Interferon alpha 5	*INF*α5	*trn*I/*trn*A	Prrn	P*psb*A/T*psb*A	ND	**21**
Interferon alpha 2b	*INF*α2B	*trn*I/*trn*A	Prrn	P*psb*A/T*psb*A	19%	**22**
Human Serum Albumin	*hsa*	*trn*I/*trn*A	Prrn^{a}, P*psb*A^{b}	ggagg^{a}, *psb*A^{b} / T*psb*A	0.02%^{a}, 11.1%^{b}	**23**
Interferon gamma	*IFN-g*	*rbc*L/*acc*D	P*psb*A	P*psb*A/T*psb*A	6%	**24**
Monoclonal antibodies	*Guy's 13*	*trn*I/*trn*A	Prrn	Ggagg / T*psb*A	ND	**25**
Anthrax protective antigen	*Pag*	*trn*I/*trn*A	Prrn	P*psb*A/T*psb*A	4-5%	**26**
Plague vaccine	*Ca*F1~*Lcr*V	*trn*I/*trn*A	Prrn	P*psb*A/T*psb*A	4.6 %	**27**

Table 3

Reporter / Selectable genes	**Organism**	**Ref**
cat- chloramphenicol acetyl transferase	a. Cucumber etioplasts^T b. Cultured tobacco cells^T	a. 28 b. 7
*uid*A - *β*-glucuronidase	Wheat leaves and calli^T	29
*aad*A- aminoglycoside adenine transferase	a. Chlamydomonas^S b. Tobacco^S	a. 30 b. 31
*npt*II – neomycin phosphotransferase	Tobacco^S	32
*aph*A-6 – aminoglycoside phosphotransferase	a. Chlamydomonas^S b. Tobacco^S	a. 33 b. 34
gfp- Green fluorescent protein	a. Tobacco and Arabidopsis^T b. Potato^S	a. 35 b. 36
*aad*A-*gfp* – fusion protein	Tobacco^S and rice^T	37
badh-Betaine aldehyde dehydrogenase	Tobacco^S	38
T- Transient expression; S- Stable integration		

Figure 1:

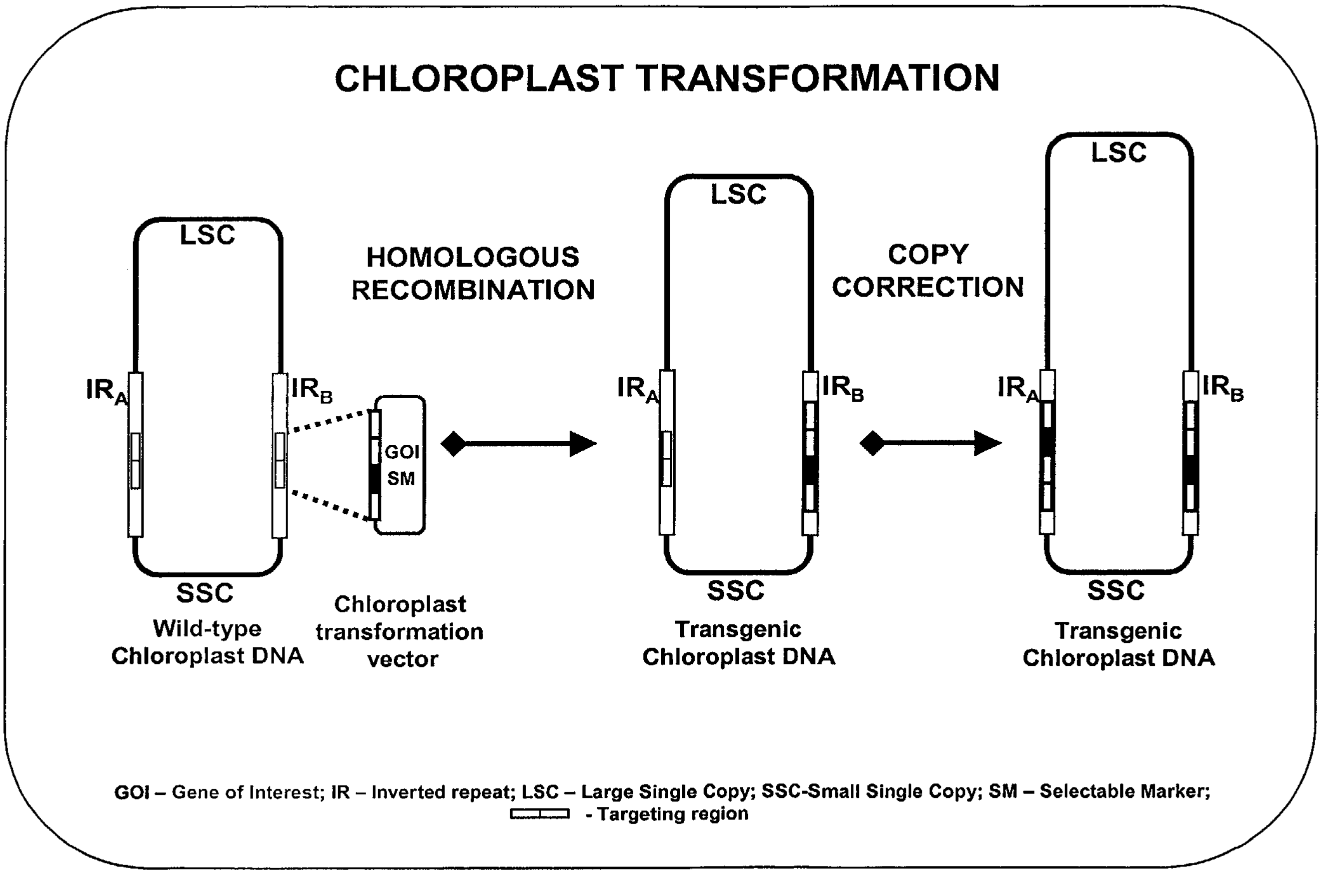

CHLOROPLAST TRANSFORMATION
LSC
IR$_A$
IR$_B$
SSC
Wild-type Chloroplast DNA
GOI
SM
Chloroplast transformation vector
HOMOLOGOUS RECOMBINATION
LSC
IR$_A$
IR$_B$
SSC
Transgenic Chloroplast DNA
COPY CORRECTION
LSC
IR$_A$
IR$_B$
SSC
Transgenic Chloroplast DNA
GOI – Gene of Interest; IR – Inverted repeat; LSC – Large Single Copy; SSC-Small Single Copy; SM – Selectable Marker;
- Targeting region

Figure 2:

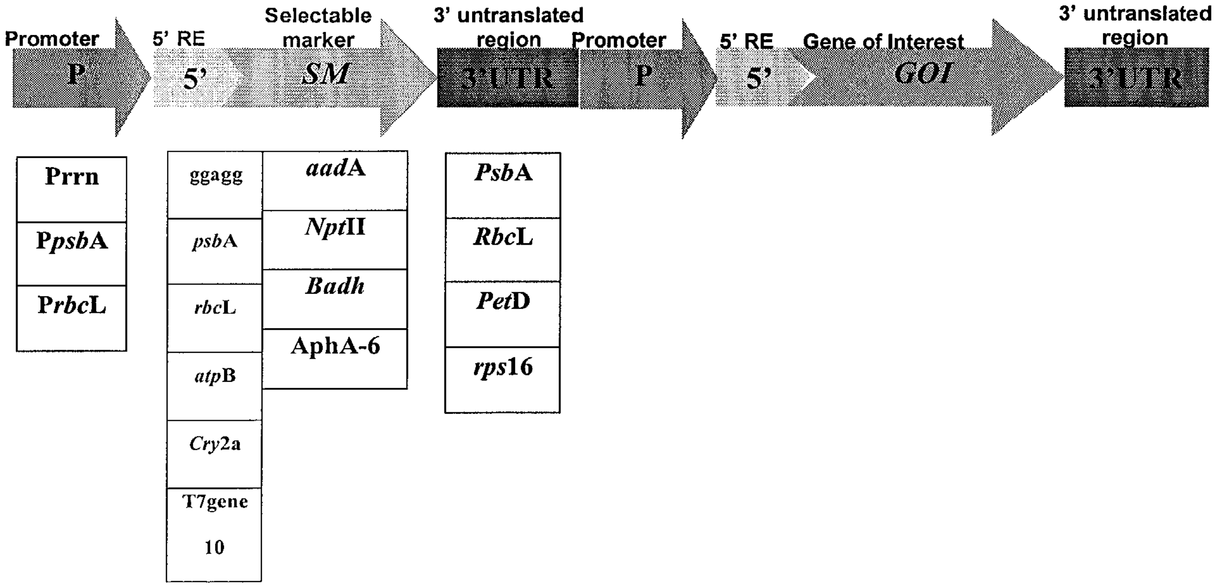
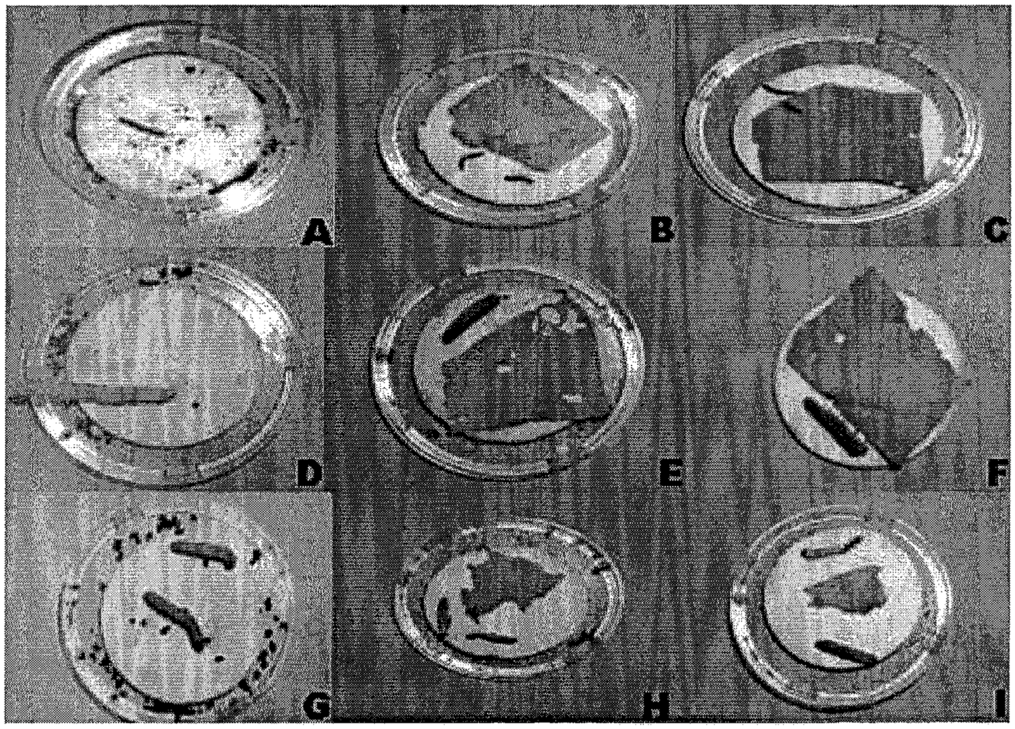

Promoter
P
5' RE
5'
Selectable marker
SM
3' untranslated region
3'UTR
Promoter
P
5' RE
5'
Gene of Interest
GOI
3' untranslated region
3'UTR
Prrn
P*psb*A
P*rbc*L
ggagg
*psb*A
*rbc*L
*atp*B
*Cry*2a
T7gene 10
*aad*A
*Npt*II
Badh
AphA-6
*Psb*A
*Rbc*L
*Pet*D
*rps*16

Figure. 3:



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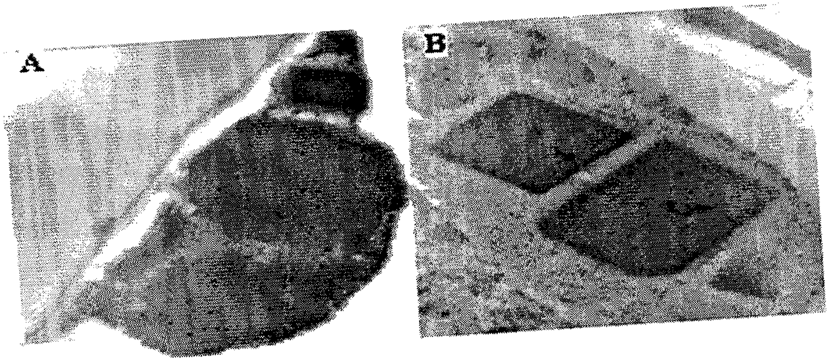

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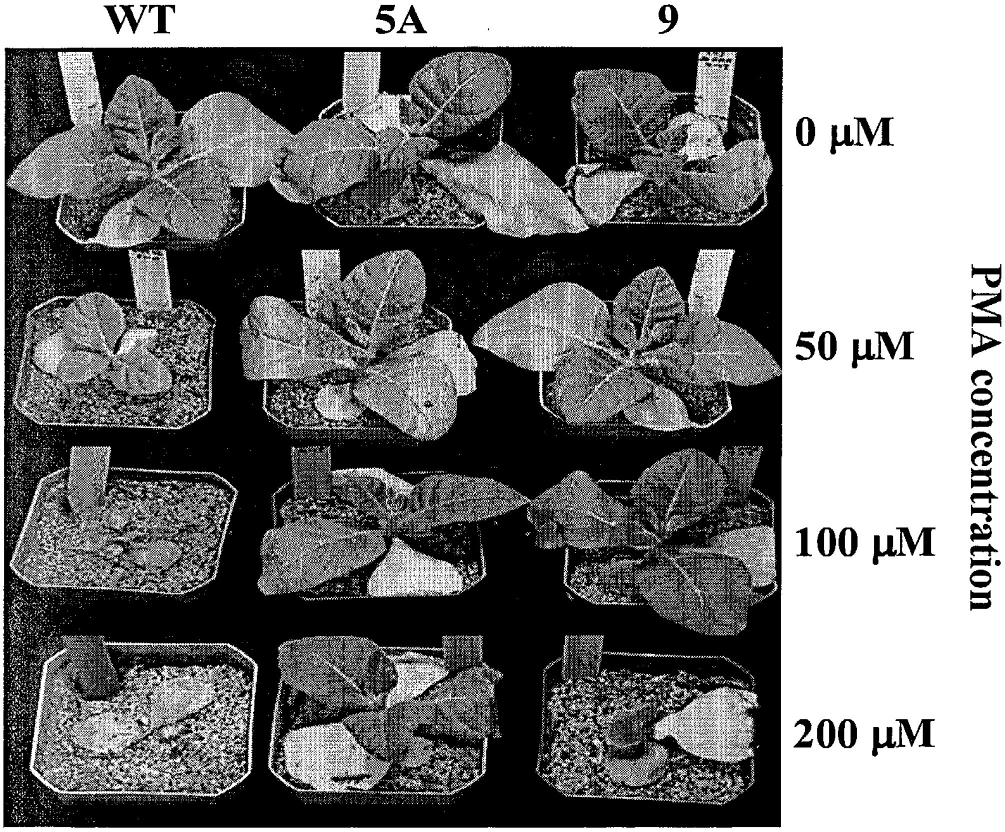

WT
5A
9
0 μM
50 μM
100 μM
200 μM
PMA concentration

Figure 6.

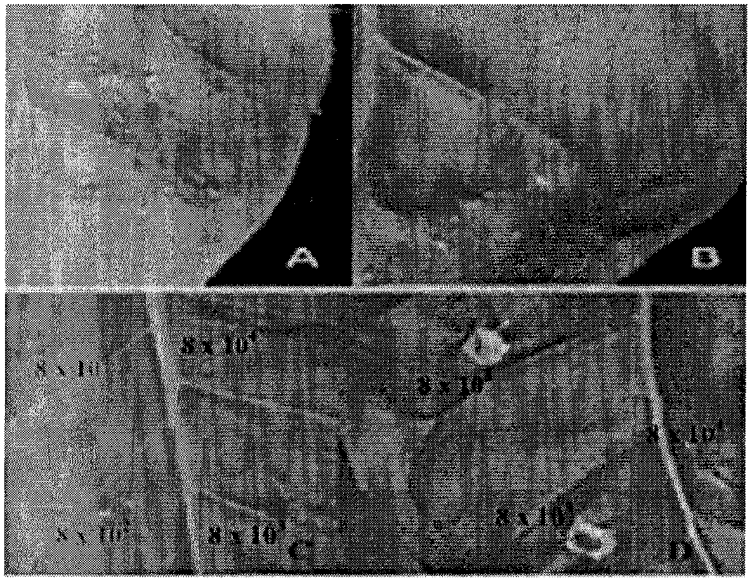
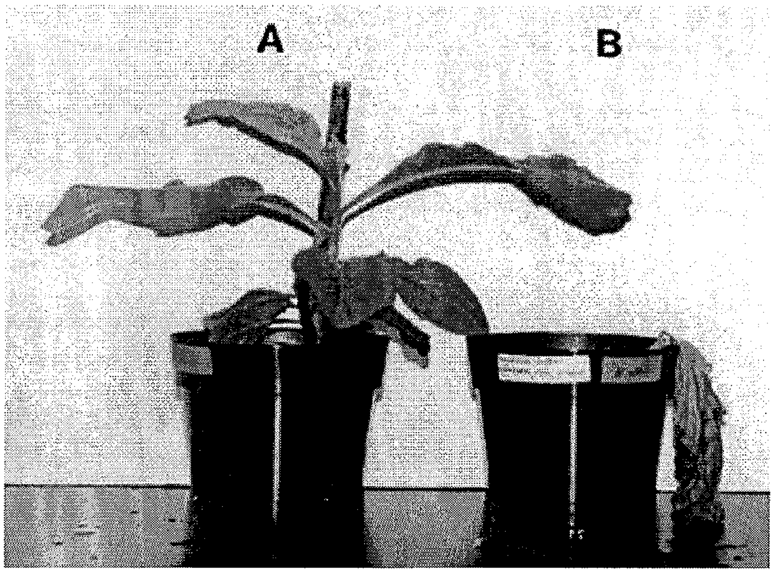

A
B
8 x 10
8 x 10
8 x 10
8 x 10
8 x 10
8 x 10
8 x 10
C
D

Figure 7.



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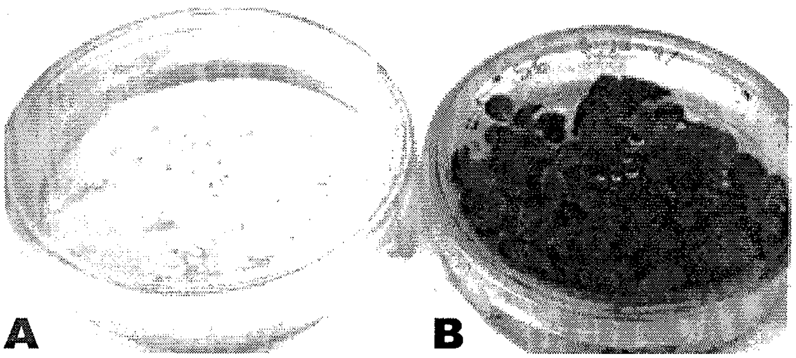

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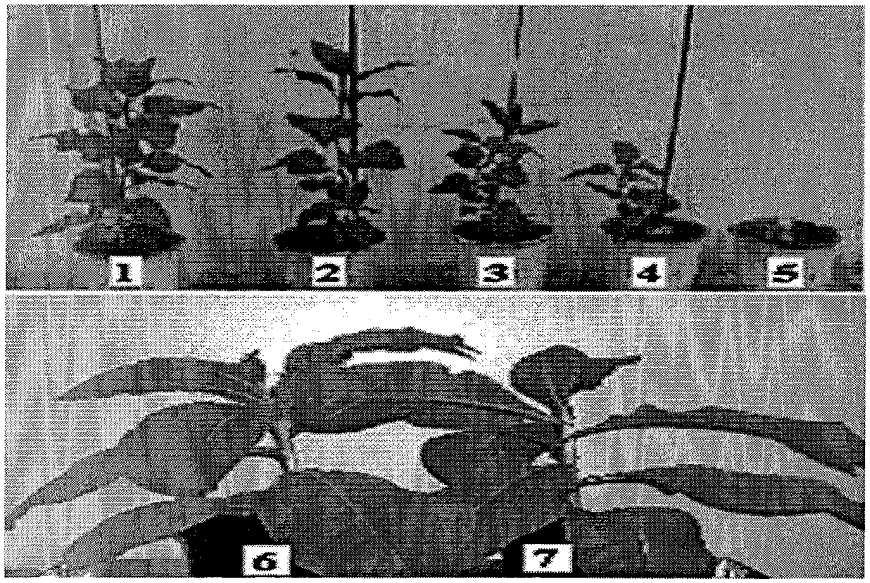
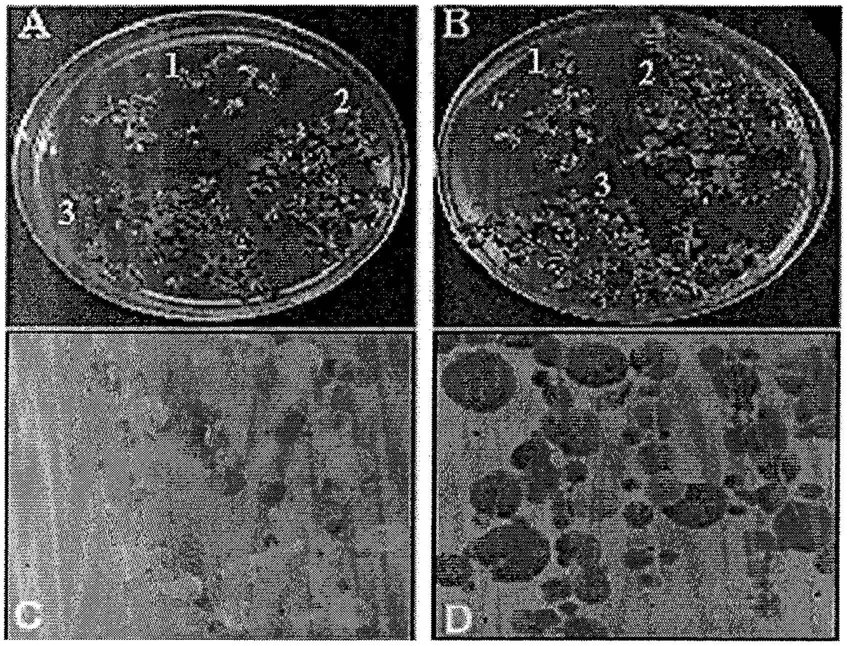


Figure 10


A
1
2
3
B
1
2
3
C
D

Tables and Figure Legends

Table 1: List of agronomic traits engineered via the chloroplast genome.

Table 2: List of biopharmaceutical proteins expressed via the chloroplast genome.

Table 3: List of reporter and selectable marker genes expressed via the chloroplast genome.

Figure 1: Schematic representation of chloroplast transformation showing the phenomenon of homologous recombination and copy correction.

Figure 2: Schematic representation of the chloroplast specific expression cassette. For a list of regulatory elements and genes of interest used for chloroplast transformation, refer to Tables 1 and 2.

Figure 3: Insect bioassays. (A,D,G) Untransformed tobacco leaves; (B,E,H) single gene-derived cry2Aa2 transformed leaves; (C,F,I) operon-derived cry2Aa2 transformed leaves. (A-C) Bioassays with *Heliothis virescens* (tobacco budworm); (D-F) bioassays with *Helicoverpa zea* (cotton bollworm); (G-I) bioassays with *Spodoptera exigua* (beet armyworm). For each replicate samples from the same leaf were used.

Figure 4: Transmission electron micrograph. A. Detection of Cry2A protein by immunogold labeling using Cry2A antibody. B. Cuboidal crystals of Cry protein in transgenic chloroplasts.

Figure 5: Effect of PMA concentration on the growth of wild type and transgenic tobacco lines. Plants were treated with 200 mL Hoagland's nutrient solution supplemented with 0, 50, 100, and 200 μM PMA. Photographs were taken 14 days after treatment. WT: negative control Petit Havana, 5A: pLDR-MerAB transgenic line, 9: pLDR-MerAB-3'UTR transgenic line.

Figure 6: *In planta* bioassays for disease resistance. A,B: Fungal disease resistance. Leaves were inoculated on the adaxial surface with 8 drops of 10 μl each of the culture containing 1 x 10^6 spores /ml of the fungal pathogen *Colletotrichum destructivum*. A. Wild type leaf; B. transgenic leaf. C,D: Bacterial disease resistance: 8×10^5, 8×10^4, 8×10^3 and 8×10^4 cell

cultures of bacterial pathogen *Pseudomonas syringae pv tabaci* (Pst) were added to a 7 mm scraped area in transgenic and non-transgenic tobacco lines (C, D). Photos were taken 5 days after inoculation.

Figure 7: Herbicide resistance assay. 18-week old chloroplast transgenic and wild type plants were sprayed with 5 mM glyphosate solution. A. Chloroplast transgenic line; B. Wild type control.

Figure 8: Maternal Inheritance of transgenes. A. Wild type and B. Chloroplast transgenic seeds expressing EPSPS gene were germinated on MSO medium supplemented with 500 mg/l spectinomycin.

Figure 9: Comparison of nuclear and chloroplast transgenic lines to illustrate pleiotropic effect. 1, Wild type tobacco; 2 – 5, T_0 nuclear transgenic lines expressing *tps*1 (2 has the lowest expression levels compared to 5); 6, T_1 chloroplast transgenic line expressing *tps*1; 7, wild type tobacco plant.

Figure 10: Dehydration / rehydration assay A, B. 3 week old seedlings were dried for 7 hours and rehydrated in MS medium for 48 hours. 1. Untransformed; 2,3. T_1 and T_2 chloroplast transgenic lines. Assays for drought tolerance. 4 week old seedlings were grown on MS medium with 6% PEG. C. Untransformed, D. T2 chloroplast transgenic line.